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Pertussis toxin (PTX) is a major virulence factor in whooping cough and can elicit protective antibodies. Amino acid residues 8 to 15 of PTX subunit S1 are important for the adenosine diphosphate–ribosyltransferase activity associated with the pathobiological effects of PTX. Furthermore, this region contains at least a portion of an epitope that elicits both toxin-neutralizing and protective antibody responses in mice. The gene encoding the S1 subunit was subjected to site-specific mutagenesis in this critical region. A mutant containing a single amino acid substitution (Arg^9→Lys) had reduced enzymatic activity (approximately 0.02% of control) while retaining the protective epitope. This analog S1 molecule may provide the basis for a genetically detoxified PTX with potential for use as a component of an acellular vaccine against whooping cough.

DESPITE THE INTRODUCTION OF AN efficacious vaccine more than 40 years ago (*1*), pertussis (or whooping cough) remains a major worldwide cause of infant morbidity and mortality. Infection accounts for approximately 60 million cases of severe respiratory tract disease and 1 million deaths annually (*2*). The etiologic agent of whooping cough is *Bordetella pertussis*, a Gram-negative nonmotile bacterium that produces a toxin having pleiotropic effects on the infected host. This pertussis toxin (PTX) is believed to occupy a central role in the disease stage of *B. pertussis* infection (*3*).

The maintenance of effective vaccination programs for pertussis has been compromised by the occurrence of untoward reactions in recipients of inactivated whole-cell vaccines (*4*). Clinical experience with acellular fractionated vaccines indicated that they reduce such side effects (*5*). These studies also demonstrated that PTX can confer protection against pertussis (*5, 6*). Unfortunately, the processes used to inactivate the toxin can significantly reduce its immunogenicity while not completely eliminating its cytopathic effects (*7*). Indeed, residual PTX activity may account for the rare occurrence of severe reactions with whole-cell vaccines that can result in permanent neurologic damage and death. The concerns regarding the relative safety of whole-cell vaccines and chemically detoxified acellular vaccines have provided an impetus to produce an immunogenic form of PTX by recombinant DNA

techniques that is free of measurable PTX activity and other potentially toxic *B. pertussis* components.

The PTX molecule is composed of five subunits: S1 (M_r 26,026), S2 (M_r 21,925), S3 (M_r 21,873), S4 (M_r 12,059), and S5 (M_r 11,013) found in a molar ratio of 1:1:1:2:1, respectively (*8*). The S1 subunit comprises the A protomer of the toxin which, like the analogous subunits of both cholera toxin and enterotoxigenic *Escherichia coli* heat-labile toxin, has adenosine diphosphate (ADP)–ribosyltransferase activity and an associated nicotinamide adenine dinucleotide (NAD) glycohydrolase activity (*9*). Substrates for PTX-catalyzed ADP-ribosylation in mammalian cells include the membrane-bound guanine nucleotide-binding regulatory proteins (N_i or G_i) of the adenylate cyclase complex (*10*). ADP-ribosylation of these target proteins leads to impaired regulation of adenosine 3′,5′-monophosphate (cAMP) formation, which may be directly responsible for the pathophysiologic effects of the toxin. The subunits S2, S3, S4, and S5 form the B oligomer of the holotoxin molecule, thought to provide mammalian cell receptor recognition and transport functions for the toxin (*11*).

The PTX operon has been cloned and its genetic organization determined (*12–14*). Several laboratories, including our own, have subcloned and expressed the individual subunit genes in *E. coli* (*15, 16*). We have now attained high-level production of the individual subunits as nonfusion proteins in *E. coli* (*17*). To elucidate the structural correlates of S1 subunit–associated enzyme activity, we produced a series of end-deleted gene fragments that encoded S1 polypeptides truncated at defined distances from the mature amino terminus (*18*). We found that deletion of the region containing amino acid residues 8 through 15 resulted in loss of

detectable enzyme activity; this region also contains at least a portion of an antigenic determinant that is recognized by a monoclonal antibody to S1 (mAb 1B7), which passively protects mice from both PTX intoxication and *B. pertussis* challenge (*19*). This region represents one of two areas of amino acid sequence homology with the A protomers of cholera toxin and *E. coli* heat-labile enterotoxin (*13*); we therefore refer to it as a homology box (*18*). Here we report that site-specific mutagenesis of the homology box yields a recombinant DNA-derived S1 subunit analog that has substantially reduced enzymatic activity, yet retains the mAb 1B7 epitope.

To effect the mutagenic alterations, we synthesized oligonucleotides (*20*) that incorporated a series of single-codon and double-codon substitution mutations within the homology box; in addition, a mutation was also designed that allowed for selective deletion of the homology region. Two previously described S1 expression vectors were used for construction of plasmids mutated in
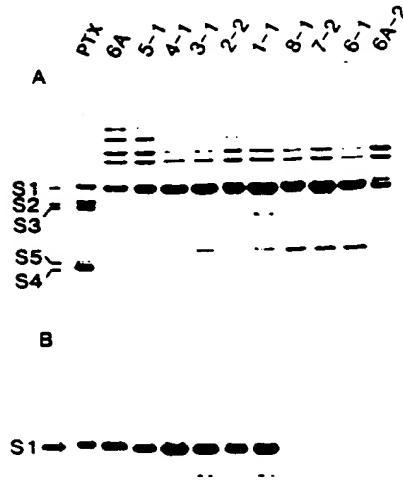
Fig. 1. SDS-PAGE and immunoblot (Western blot) analysis of mutant S1 proteins. Inclusion body preparations were obtained from recombinant *E. coli* by French-press lysis and differential centrifugation (*17*), the inclusions solubilized in electrophoresis sample buffer, and analyzed by electrophoresis in 15% acrylamide gels containing SDS (*21*). (**A**) Each S1 mutant preparation (5 μg) and purified PTX (5 μg) were analyzed; the proteins were stained with Coomassie brilliant blue R-250. (**B**) Each S1 mutant preparation (2.5 μg) and purified PTX (2.5 μg) were analyzed; the proteins were electrophoretically transferred to a nitrocellulose sheet (*22*) that was subsequently incubated with 1B7, a mAb to S1 (*19*). Bound antibody was detected with rabbit antibody to mouse immunoglobulin G (IgG) coupled to horseradish peroxidase and 4-chloro-1-naphthol as the chromogenic substrate.

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the homology box: pPTXS1/6A and pPTXS1/33B (18). S1/6A is an S1 analog in which the mature amino-terminal aspartyl-aspartate is replaced with methionylvaline; S1/33B has its first 14 amino acid residues replaced with methionylvaline. Both enzymatic activity and mAb 1B7 reactivity are retained in S1/6A, whereas S1/33B has neither (18). We constructed the expression vector for each S1 substitution mutant in a three-way ligation using the appropriate oligonucleotide with Acc I and Bsp MII cohesive ends, an 1324-bp DNA fragment from pPTXS1/6A (Acc I–Sst I), and a 3.56-kb

DNA fragment from pPTXS1/33B (Bsp MII–Sst I). The ligation and the relatively short length of the oligonucleotides required for the substitutions was facilitated by the presence of novel Bsp MII and Nla IV restriction sites generated in the original construction of pPTXS1/33B (18). Deletion of the homology box involved ligation of a mung bean nuclease–blunted Acc I site to the left of the box in pPTXS1/6A, and an Nla IV site to the right of the box in S1/33B; this ligation resulted in the excision of codons for Tyr^8 through Pro^{14}. Vector construction and retention of the altered

sites were confirmed by restriction analysis and partial DNA sequence analysis. The specific codon changes that were used, and the resultant amino acid substitutions, are shown in Table 1.

The expression vector constructions were transformed into E. coli, and the mutant S1 genes were expressed after temperature induction. In this expression system (17), the recombinant S1 polypeptides are synthesized at high phenotypic levels (7 to 22% of total cell protein) and segregated into intracellular inclusions. Inclusion bodies were recovered after cell lysis (17) and examined by SDS–polyacrylamide gel electrophoresis (PAGE) (21) (Fig. 1A). The electrophoretic profile revealed that the mutagenized S1 products constituted the predominant protein species in each preparation and that their mobilities were very similar to that of the parent S1/6A subunit.

To examine the phenotypic effects of the mutations on antigenicity, we assayed the mutant S1 polypeptides for their ability to react with the protective mAb 1B7 in an immunoblot format (22). The parent construction 6A and each of the single-codon substitution mutants (5-1, 4-1, 3-1, 2-2, and 1-1) retained reactivity with mAb 1B7 (Fig. 1B). In contrast, the reactivity of those mutants containing double-residue substitutions (8-1, 7-2, and 6-1), as well as the mutant in which the homology box had been deleted (6A-2), was significantly diminished or abolished. If this protective S1 epitope is to be retained in a subsequent vaccine preparation, only those analogs with single substitutions in the homology box would be potentially useful.

The mutant S1 molecules were assayed for ADP-ribosyltransferase activity as measured by the transfer of radiolabeled ADP-ribose from [adenylate-^{32}P]NAD to purified bovine transducin (23), a guanine nucleotide-binding regulatory protein found in the rod outer segment membranes (24). As shown in Table 1, each of the substitutions appeared to reduce specific ADP-ribosyltransferase activity, with the exception of mutants 5-1 and 2-2, which retained the full activity associated with the parent 6A species; 6A has approximately 60% of the ADP-ribosyltransferase activity of authentic S1 (18). Neither mutant 4-1 nor any of the double-substitution mutants exhibited any significant transferase activity when compared to the inclusion body protein control (denoted 20A); this control is a polypeptide of M_r 21,678, derived from a major alternative open reading frame (orf) in the S1 gene and does not contain S1 subunit-related sequences.

The most noteworthy S1 analog produced was 4-1 (Arg^9→Lys). It alone among
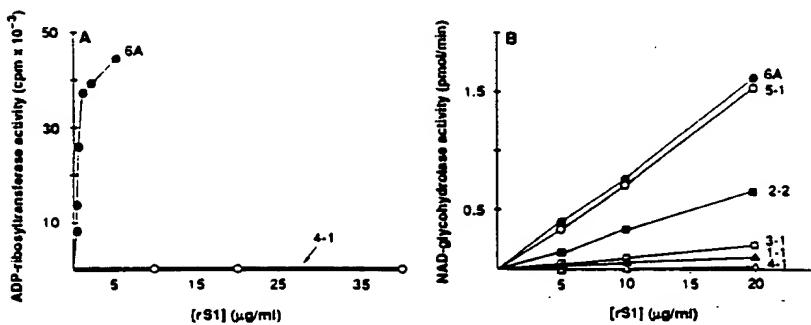
Fig. 2. ADP-ribosyltransferase and NAD-glycohydrolase activity of recombinant analog S1 proteins containing single amino acid substitutions. (A) Recombinant S1 (rS1) subunits 6A and analog 4-1 assayed as described in the legend to Table 1. The values obtained for mutant 4-1 were 0, 141, and 72 cpm at 10, 20, and 40 μg/ml, respectively. (B) NAD-glycohydrolase activity of rS1/6A and each single-residue mutant measured as the release of [^{14}C]nicotinamide from [carbonyl-^{14}C]NAD (18). In both assays, each data point represents the mean of duplicate determinations from which the value for the negative control (inclusion-body preparation of orf mutant 20A) was subtracted.

Table 1. ADP-ribosyltransferase activity of recombinant S1 mutant polypeptides. Intracellular inclusions containing the recombinant subunits produced in E. coli were recovered by differential centrifugation and extracted with 8M urea (18). The urea extracts were adjusted to a total protein concentration of 0.6 mg/ml, dialyzed against 50 mM tris-HCl (pH 8.0), and then centrifuged at 14,000g for 30 min. The amount of recombinant product in the supernatant fractions was determined by quantitative densitometric scanning of proteins separated by SDS-PAGE and stained with Coomassie blue. ADP-ribosyltransferase activity was determined (17) with the use of 4.0 μg of purified bovine transducin and 100 ng of each S1 analog. The values represent the transfer of [^{32}P]ADP-ribose to the α subunit of transducin, as measured by total trichloroacetic acid–precipitable radioactivity, and each is given as the mean of triplicate determinations with standard deviation. The 20A product represents a negative control because its synthesis results in the formation of intracellular inclusions that lack S1-related proteins.

Mutant designation	Amino acid change	Codon change	ADP-ribosyltransferase activity (cpm)
6A	None	None	23,450 ± 950
5-1	Tyr^8 → Phe	TAC → TTC	26,361 ± 1,321
4-1	Arg^9 → Lys	CGC → AAG	754 ± 7
3-1	Asp^{11} → Glu	GAC → GAA	13,549 ± 1,596
2-2	Ser^{12} → Gly	TCC → GGC	22,319 ± 2,096
1-1	Arg^{13} → Lys	CGC → AAG	7,393 ± 1,367
8-1	Tyr^8 → Leu / Arg^9 → Glu	TAC → TTG / CGC → GAA	926 ± 205
7-2	Arg^9 → Asn / Ser^{12} → Gly	CGC → AAC / TCC → GGC	753 ± 30
6-1	Asp^{11} → Pro / Pro^{14} → Asp	GAC → CCG / CCG → GAC	764 ± 120
20A	Alternate S1 orf	—	839 ± 68

the single-substitution mutants exhibited little or no transferase activity under the conditions we used (Table 1); however, unlike the double mutants, it retained reactivity with neutralizing mAb 1B7. Only small amounts of enzymatic activity could be detected when the amount of 4-1 protein in the assay was increased (Fig. 2A); repeated determinations indicated that the specific ADP-ribosyltransferase activity of the S1 analog was reduced by a factor of approximately 5000. Measurement of the NAD glycohydrolase activity associated with the single-residue substitution mutants (Fig. 2B) revealed a pattern similar to that obtained from evaluation of ADP-ribosyltransferase activity. The S1 analog 4-1 exhibited little or no detectable glycohydrolase activity, indicating a reduction in the magnitude of this activity by a factor of 50 to 100.

The results obtained here substantiate our previous conclusion (18): the region of S1 homology (residues 8 to 15) with cholera and E. coli toxins contains amino acids that are important for enzyme activity. The magnitude of the effect exerted by substitution of Arg^9 on both transferase and glycohydrolase activities suggests that this residue plays an essential role in the enzymatic mechanisms of the S1 subunit. We recently introduced the Arg^9→Lys mutation into full-length recombinant S1 and found that transferase activity was reduced by a factor of approximately 1000. This observation indicates that the substitution at residue 9 is alone sufficient to attain the striking loss in enzyme activity and that the coincidental replacement of the two amino-terminal aspartate residues in the mature S1 sequence with the Met-Val dipeptide that occurs in S1/6A is not required to achieve this reduction.

Inactivation of enzymatic and toxic activity through single amino acid substitutions effected by site-directed mutagenesis has been achieved for both Pseudomonas exotoxin A and diphtheria toxin (DTX) (25). In addition, Porro et al. (26) have shown that an enzymatically inactive mutant form (CRM197) of DTX elicits a protective response. These studies support the utility of "genetic toxoiding" and have prompted similar efforts with respect to PTX. Black et al. (27) have recently described a strain of B. pertussis that produces a mutant form of PTX with reduced ADP-ribosyltransferase activity. The strain showed little or no evidence of PTX-mediated biological activity (for example, lymphocytosis or histamine sensitization) when injected into mice, thereby supporting the prediction that suppression of S1-associated enzyme activity will result in a corresponding reduction in toxic effects. However, the mutant strain also had diminished immunoprotective capability when used in the intracerebral challenge model and this diminished capability was interpreted to reflect an immunopotentiating effect of the ADP-ribosyltransferase activity. It will be of interest to evaluate the effects of the 4-1 mutation that retains reactivity with a known protective mAb on the toxic and immunogenic properties of the holotoxin.

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